



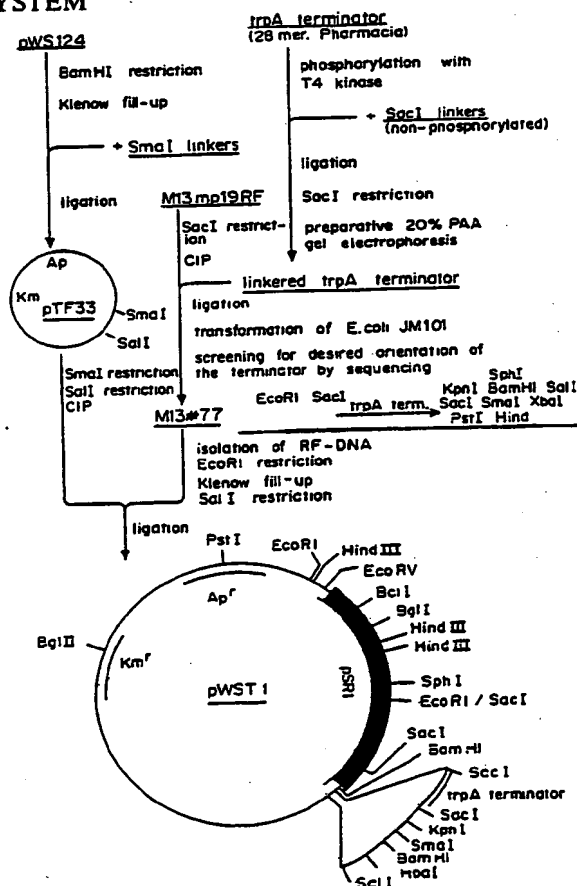
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(71) Applicant: MASSACHUSETTS INSTITUTE OF TECHNOLOGY [US/US]; 77 Massachusetts Avenue, Cambridge, MA 02139 (US). (72) Inventors: LIEBL, Wolfgang ; Hart Str. 17, D-8057 Echling (DE). SINSKEY, Anthony, J. ; 285 Commonwealth Avenue, Boston, MA 02115 (US).		Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>

(54) Title: CORYNEFORM EXPRESSION AND SECRETION SYSTEM

(57) Abstract

A coryneform expression and secretion system for homologous and heterologous genes consisting of the host, nucleotide sequences encoding a protein of interest, signals for expression and, optionally, targeting signals which direct membrane anchoring and for secretion and processing of the expressed protein. Regulatory signals may be utilized to control the rate and extent of expression and secretion. The system may further include compounds such as ionophores for altering the membrane transport of the host. The host itself may be mutated to alter transport, for example, by decreasing the mycolic acid content of *Corynebacteria* species. The preferred host is a *Corynebacterium* although other coryneforms deficient in extracellular protease production may also be used. *C. glutamicum* is used as a model organism for the secretion system. This Gram positive, non-pathogenic bacteria can efficiently utilize heterologous expression and secretion signals originating from a variety of both Gram negative and Gram positive bacteria to provide the basis for the overproduction and secretion of cloned gene products in a given *Corynebacterium* host, as demonstrated by the expression and secretion by *C. glutamicum* of a lipase encoded by a gene from *S. hyicus* and a thermonuclease encoded by a gene from *S. aureus*.



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CORYNEFORM EXPRESSION AND SECRETION SYSTEMBackground of the Invention

The present invention is in the area of improved procaryotic expression systems and, in particular, a Coryneform host system for the expression and excretion of gene products.

Coryneform bacteria are a taxonomically ill-defined group of Gram positive bacteria originally related by unique morphological features. These microorganisms occupy a wide variety of ecological niches and display an even broader array of interesting and useful properties. With the advent of systematic chemical analysis, there is considerable evidence indicating that the genus Corynebacterium is closely related to Mycobacterium and Norcardia. Included in the genus Corynebacterium are medically important species such as C. diphtheriae, animal pathogens such as C. renale, plant pathogens and diverse saprophytic, aerobic coryneform bacteria. The saprophytic coryneform bacteria are widely distributed in nature and include not only Corynebacterium species but also other bacteria including Arthrobacter, Brevibacterium, Cellulomonas, Microbacterium and Curtobacterium. The coryneform group thus represents an important source of enzymes, primary metabolites, and genetic material.

When cloning heterologous proteins for purification, it is often desirable to have the gene product hyperproduced and/or secreted by the host cells. The major advantages of secretion over

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intracellular accumulation of recombinant proteins are an increase in yield and the facilitation of product purification. Translocation of proteins into or through membranes is an essential feature of prokaryotic and eukaryotic cells. Proteins that are partially or fully integrated into membranes, proteins that are associated or covalently bound to cell walls, or proteins that are secreted, must cross the cytoplasmic membrane.

Although initial investigations on protein export have been carried out with eukaryotic systems, there is an increasing interest in the mechanism and genetics of bacterial protein export. Benson et al., Cell 32, 1325-1335 (1985); D. Oliver, Ann.Rev.Microbiol. 39, 615-648 (1985); Randall and Hardy, Microbiol.Rev. 48, 290-298 (1984); and Pugsley and Schwartz, FEMS Microbiol.Rev. 48, 290-298 (1985), have recently reviewed this area. The Gram negative E. coli is the best-studied species among the prokaryotes. The most advanced experimental techniques have been tailored especially to fit the E. coli system. Despite the fact that the Gram positive cell wall has a simpler structure than its Gram negative counterpart, that Gram positive organisms are often very efficient in secreting proteins to the culture medium as compared with Gram negative organisms which normally cannot transport proteins beyond the outer membrane of their cell envelope, and that a vast number of extracellular proteins of Gram positive bacteria have been isolated and examined, including most bacterial

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enzymes of commercial importance, the use of these organisms for basic investigations of protein export has been limited.

5 Most exported proteins, contrary to the majority of proteins localized in the cytoplasmic membrane, are synthesized as precursors with an N-terminal peptide extension (signal peptide) that is cleaved off in the course of translocation. Many of the bacterial and eukaryotic signal sequences
10 that have been studied share striking structural similarities and are in fact interchangeable, as reported by several investigators. For example, the E. coli leader peptidase precisely recognizes and cleaves eukaryotic precursors.

15 Protein fusion experiments have demonstrated that a signal sequence alone is generally insufficient for the proper export of proteins. Several other types of targeting signals in addition to signal peptides have been identified. The most
20 complex situation is found in eukaryotic cells where proteins must be directed to different subcellular compartments: endoplasmic reticulum, mitochondria, or chloroplasts. Additional information in the body of the mature protein may also be necessary. For
25 example, posttranslational modification may contribute to the final localization of a protein, as seen with Gram negative lipoproteins and Gram positive lipopenicillinases.

Unfortunately, at this time, a good Gram
30 positive cloning host has not been identified. The classic Gram positive cloning host, B. subtilis,

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secretes extracellular proteases which attack heterologous proteins expressed in this organism, as reported by Ulmanen et al., J.Bacteriol. 162,176-182 (1985) and Doi et al., Trends in Biotech. 232-235 (1986). Consequently, there is a clear need for alternative Gram positive host organisms.

Protein secretion by coryneform bacteria has not been investigated, other than the secretion of diptheria toxin by the pathogenic C. diptheriae upon infection with certain lysogenic tox⁺ phages, reported by Pappenheimer, Ann.Rev.Biochem. 46,69-94 (1977) and Neville and Hudson, Ann.Rev.Biochem. 55,195-224 (1986). Even reports of the cloning in Corynebacterium hosts of the genes for two proteins which are normally exported in their native hosts, beta-lactamase from E. coli and alpha-amylase from Bacillus amyloliquefaciens, do not disclose whether or not these heterologous proteins were secreted.

It is therefore an object of the present invention to provide a Gram positive bacterial expression and secretion system.

It is another object of the present invention to characterize gene expression (replication, conjugal transfer and plasmid biology), in the Gram positive bacterial expression system.

It is yet another object of the present invention to further elucidate the genomic organization and structure of the Gram positive host, including the isolation and characterization of high efficiency and regulatable promoters.

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Summary of the Invention

A Coryneform expression and secretion system for cloned gene sequences consisting of the host nucleotide sequences encoding a protein of interest, signals for expression and, optionally, targeting signals which direct membrane anchoring and for secretion and processing of the expressed protein. In the preferred embodiment of the system for the expression and secretion of homologous and heterologous sequences, a Corynebacteria is selected as the host. The protein-encoding gene, transcriptional and translational start signals and a sequence coding for a secretion signal peptide are usually provided in a secretion vector, optionally including an inducible promoter. Regulatory signals may be utilized to control the rate and extent of expression and secretion. The system may further include compounds such as ionophores for altering the membrane transport of the host. The host itself may be mutated to alter transport, for example, by decreasing the mycolic acid content.

C. glutamicum is used as a model organism for the secretion system. This is a Gram positive, non-pathogenic bacterium which can efficiently utilize heterologous expression and secretion signals originating from a variety of both Gram negative and Gram positive bacteria to provide the basis for the overproduction and secretion of cloned gene products in a given Corynebacterium host.

The overexpression and secretion of foreign cloned genes in this system, and the exceptional

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suitability of Corynebacterium as a host organism for the production and secretion of foreign gene products, is demonstrated by the expression and secretion by C. glutamicum of a lipase encoded by a gene from S. hyicus and a thermonuclease encoded by a gene from S. aureus.

Brief Description of the Drawings

Figure 1 is the construction of plasmid pWST1. A SmaI restriction site was introduced into the BamHI site of pWS124 on a synthetic oligonucleotide to create pTF33. The trpA terminator, from E.coli, was obtained as a 28 bp oligonucleotide and ligated to SacI linkers and cloned into M13 mp19 and screened by sequencing. Replicative form DNA of M13#77 was isolated and the terminator introduced into the SmaI-SacI region of pTF33 as a blunt ended EcoRI-SacI fragment to create pWST1.

Figure 2 is the restriction endonuclease map of pGN1. The C. glutamicum DNase gene was shown to reside on a 1.1 kb BamHI-BclI fragment within the 2.7 kb chromosomal DNA insert.

Detailed Description of the Invention

The present invention is a Coryneform expression and secretion system, and methods for its manufacture, demonstrated using C. glutamicum as a model organism for this group.

As discussed in the Background of the Invention, the coryneform group of bacteria encompasses a

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number of diverse, Gram positive organisms, including Corynebacterium, Arthrobacteria, Nocardia, Mycobacterium, Cellulomonas, Microbacterium and Curto-
5 bacterium. To be useful in the present invention, the bacterial host must be deficient in protease production, especially extracellular proteases. Preferably, the host should be amenable to large scale fermentation conditions, i.e., have simple
10 nutritional requirements; grow within a wide range of temperatures, between 20 and 65°C; of food grade or being generally regarded as safe, such as Brevibacterium lactofermentum, Lactobaccillus species and Streptococci; non-spore forming, unlike most strains of Bacillus; transformable; and genetically stable,
15 as opposed to many Bacillus and Streptomyces strain. Corynebacteria strains and particularly C. glutamicum, are preferred at this time.

In the past, it has not been possible to directly address questions of gene organization,
20 structure and regulation of Corynebacterium at the molecular level due to deficiencies in the genetic tools that were available, even though Coryne-
bacterium has long occupied a central role in the manufacture of a variety of primary metabolites
25 including L-amino acids, nucleotides and organic acids by conventional fermentation. Several features of C. glutamicum make it especially desirable for extensive genetic studies: C. glutamicum is a nonpathogenic, food-grade microorganism, and the
30 biochemistry and enzymology of C. glutamicum biosynthetic pathways have been extensively characterized.

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As detailed below, there are a number of methods and variables to consider in the construction of an expression system using Corynebacterium as the host. Depending on the protein to expressed, the degree of regulation and quantity of expression desired, and whether or not the protein is to be secreted and/or targeted, the system can consist of as little as the host and an expression vector containing transcription and translation signals and the gene to be expressed. For the expressed protein to be secreted, the vector must further include targeting signals such as secretion signals, processing signals, and membrane anchoring signals. For control of the rate and extent of expression, the promotor may be placed under the control of a repressor or stimulatory protein. The expression/secretion system as a whole may also be modified by point mutations or deletions in any of the sequences discussed above, by the addition of membrane transport altering substances, or by mutations in the host affecting cell components such as the mycolic acid content of the cell wall.

The first consideration is to determine the protein to be expressed and to isolate a sequence encoding some or all of the protein. Methods for isolation of protein-encoding sequences are known to those skilled in the art of genetic engineering. Examples of the isolation and characterization of genes from C. glutamicum are provided.

The second consideration is the selection of an appropriate vector. A useful vector is pWST1 which

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contains the E. coli trpA transcription terminator obtained from Pharmacia Fine Chemicals, Piscataway, New Jersey, immediately upstream of a polylinker cloning site. pWST1 is shown in Figure 1 and has particular utility in investigating promoter structure and function by eliminating read-through transcription from upstream promoters located within the cloning vector. Further examples of suitable vectors for use in Corynebacterium are described by U.S. Patent No. 4,649,119 to Sinskey et al. Other useful vectors, and methods for inserting the gene of interest into the vector, will be apparent to those skilled in the art.

Once the nucleotide sequence and the vector have been selected, sequences for the expression, regulation of expression, and post-translational characteristics of the expressed protein can be isolated and inserted into the vector. A number of promoters are useful in the present invention, including heterologous expression signals originating from a variety of both Gram negative and Gram positive bacteria including Escherichia, Bacillus, Staphylococcus and Streptococcus species. Of course, expression signals present in Corynebacterium are also useful. Indeed, an important feature of the present invention is not only the expression and secretion of gene products, but utilization of either homologous or heterologous gene sequences by the host.

An important element of the mechanism by which C. glutamicum mediates gene expression is through

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promoter structure and the structure/function relationships. The promoters of naturally occurring genes can be determined by S1 nuclease mapping, described by Berk and Sharp in Cell, 12, 721(1977) using comparisons between known promoter sequences and the sequence which is being characterized. Identification of the transcription initiation sites and the alignment of upstream sequences can be used to identify consensus sequences. Deletion analysis of the isolated gene can also be used to confirm the promoter identification and to allow promoter replacement. In vitro generated deletions can be constructed using restriction enzymes or the exonuclease BAL31. The insertion of linkers facilitates subsequent cloning and sequencing. Restriction enzymes and linkers are commercially available from sources including Boehringer-Mannheim Biochemicals, Indianapolis, IN, and New England Biolabs Inc., Beverly, MA.

In some situations, it may be desirable to bring expression of a gene under control of a specific effector or repressor. In this case, either a regulatable promoter could be inserted prior to the gene, or the gene fused to the 3' end of a gene under the transcriptional control of a particular metabolite such as phenylalanine. Alternatively, an inducible promoter that allows product formation to be switched on during the appropriate growth phase, for example, as described Ghrayeb et al., EMBO J. 3,2437-2442 (1984), or mutant high copy vectors that allow overproduction of proteins could be used.

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Secretion vectors are a specialized form of expression vectors and have to be specifically designed for each cloning system. They must carry efficient transcriptional and translational start signals as well as sequences coding for the N-terminal portion (at least the signal peptide) of a secreted protein. Secretion vectors have been constructed for various microbial cloning hosts, including E. coli, as reported by Takahara et al., J.Biol.Chem. 260, 2670-2674 (1985), B. subtilis, as reported by Palva et al., Proc.Natl.Acad.Sci.USA 79,5582-5586 (1982); Ohmura et al., J.Biochem. 95, 98-93 (1983); and Kovacevic et al., J.Bact. 162,521-528 (1985), staphylococci, as reported by Nilsson et al., Nucl. Acids Res. 13,1151-1162 (1985), and Liebl and Gotz, Mol.Gen.Genet. 204,166-173 (1986), and yeast, reported by Smith et al., Science 229,1219-1224 (1985).

A secretion signal sequence that functions in C. glutamicum can be selected by screening for expression of homologous or heterologous exoprotein genes in C. glutamicum and then used to form the basis of a secretion vector. The efficiency of this system can be tested by in-frame fusions of foreign genes, such as the structural gene for E. coli beta-lactamase devoid of its own expression/secretion signals, to the signal sequence. Expression of the gene fusion can be regulated either by the native promoter of the chosen exoprotein gene or by a homologous Corynebacterium promoter isolated in promoter search experiments. The amount and integrity of foreign protein released to the growth

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medium upon transformation of C. glutamicum with the gene fusion demonstrates the capacity of this host organism to produce and secrete recombinant proteins.

5 Additionally, a sequence encoding an affinity tail, such as the IgG binding domain of protein A, could be inserted into the vector following the gene to be expressed to facilitate purification of the secreted product (Nilsson et al., Nucl. Acids Res.
10 13, 1151-1162, (1985)).

 An element to be considered in protein secretion by C. glutamicum is the determination and characterization of targeting sequences which direct secretion membrane anchoring and post-translational
15 processing of protein in C. glutamicum. One source of targeting signals are extracellular enzymes such as the lipase produced by P. acnes. Cloning of this lipase gene provides an exoprotein encoding gene from a species which is relatively closely related
20 to C. glutamicum. Expression of the gene is detected by the presence of lipase activity. Staphylococci produce a number of extracellular proteins, some of which are bound to the cell wall of their native host. Examples of extracellular
25 proteins produced by S. aureus are thermonuclease and protein A, both structurally and functionally well characterized proteins. The nucleotide sequences of the genes, as well as the N-terminal amino acid sequences of the mature proteins, are
30 known. These genes can be placed under the transcriptional control of strong host promoters on an

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/01999

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶

According to International Patent Classification (IPC) or to both National Classification and IPC
 IPC(4): C12P 21/00; C12N 15/00; C12N 1/20
 U.S. 435/68; 435/172.3, 172.1, 435/253

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
U.S.	435/68, 172.3, 253, 320, 172.1 935/29, 48

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁸

Chemical Abstracts Data Base (CAS) 1967-1988.
 Biological Abstracts Data Base (BIOSIS) 1967-1988.
 Keywords: Protease mutant; signal sequence, vector plasmid.

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y, E	US, A, 4,758,512 (GOLDBERG ET AL.) 19 July 1988 See entire document particularly columns 1, 2 and 6.	1-33
Y, P	US, A, 4,745,069 (MAYNE ET AL.) 17 May 1988 See entire document, particularly columns 1-7.	1-33
Y, P	US, A, 4,745,056 (GUTERMAN ET AL.) 17 May 1988 See entire document, particularly columns 1-3.	1-33
Y, P	US, A, 4,711,844 (CHANG) 8 December 1987, See entire document particularly columns 1-8.	1-33
Y, P	US, A, 4,711,843 (CHANG), 8 December 1987 See entire document particularly columns 3 and 4.	1-33

^{*} Special categories of cited documents: ¹⁰

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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IV. CERTIFICATION

Date of the Actual Completion of the International Search

06 September 1988

Date of Mailing of this International Search Report

J 7 OCT 1988

International Searching Authority

ISA/US

Signature of Authorized Officer

THOMAS D. MAYS

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	<u>Applied and Environmental Microbiology</u> , (Washington, D.C., U.S.A) Volume 53, issued February 1987 (FAHNESTOCK ET AL.), "Protease deficient <u>Bacillus subtilis</u> host strains for production of Staphylococcal protein A". See pages 379-384, particularly pages 374 and 375.	1-33
Y	<u>Journal of Bacteriology</u> (Washington, D.C. USA), Volume 167, issued August 1986 (FOLLETTIE ET AL.) "Molecular cloning and nucleotide sequence of the <u>Corynebacterium glutamicum pheA</u> gene". See pages 695-702, particularly pages 695 and 696.	1-33
Y	<u>Journal of Bacteriology</u> , (Washington, D.C. USA) Volume 162, issued May 1985 (YOSHIHAMA ET AL.) "Cloning vector system for <u>Corynebacterium glutamicum</u> ". See pages 591-597, particularly, 591-593.	1-33